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Application of capillary electrophoresis to determination of enzyme activity and other properties of phosphatidylinositol-specific phospholipase C

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Abstract

This paper describes a method for monitoring enzymatic activity using micellar electrokinetic chromatography (MEKC). MEKC is used to analyze the activity of phosphatidylinositol-specific phospholipase C (PI–PLC) with convenience and precision. PI specific PLC enzyme converts phosphatidylinositol (PI) to diacylglycerol (DAG) and inositol 1,2-cyclic phosphate (IP). The assay system developed is based on monitoring both the breakdown of substrate and the formation of products simultaneously.

To obtain the best separation for the substrate PI and product DAG, we investigated changes in concentration of electrolyte buffer and SDS as well as in pH of the eletrolyte buffer. Since the structures of the substrate and products are different, the reaction could be monitored easily by MEKC mostly based on hydrophobicity. Under the separation conditions developed, we investigated the enzymatic activity of PI–PLC depending on the concentrations of phosphatidylinositol, various divalent cations and the reaction temperature. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF–MS) was used to confirm the molecular masses of the substrate PI and products DAG and IP.

The results of this study show that capillary electrophoresis can be widely applied to analyze and characterize many other enzymes since capillary electrophoresis has several advantages as follows, simplicity, speed, no need for radiolabelled substrate, small sample volumes and sufficient accuracy. © 1997 Elsevier Science B.V.

Keywords: Phospholipases; Enzymes; Phosphatidylinositol; Phospholipids

1. Introduction

Phospholipids are major components of cellular membranes and various subcellular organelles. These compounds give structural continuity and macromolecular functions to cellular membranes. They are also involved in signal transduction processes of various biological systems [1–3].

Phospholipid structures consist of 1,2-diacyl, acylalkyl- or dialkylglycerol esterified to phosphate which is linked to a variety of polar groups such as choline, ethanolamine, inositol and so on. The structures of the acyl and alkyl chains vary considerably, both in chain length and the degree of unsaturation.

Phospholipase C plays a pivotal role in the signal transduction process via a variety of growth factors, underlining the importance of this enzyme in growth-related pathologies such as cancer [4]. As indicated by its name, phosphatidylinositol-specific phospholipase C catalyzes the hydrolysis of phospha-

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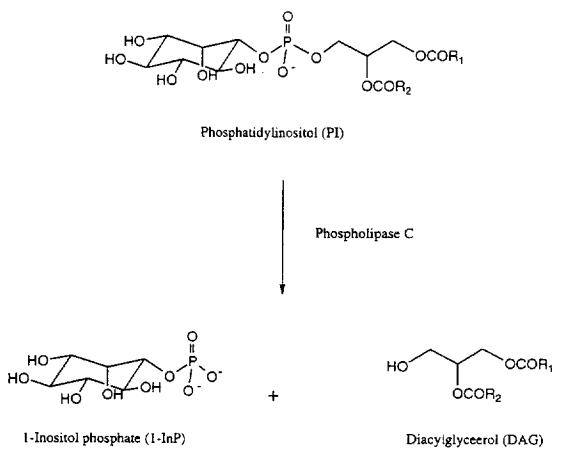

Fig. 1. Phospholipase C reaction.

tidylinositols to yield two second messenger molecules, diacylglycerol (DAG) and inositol phosphates (IP) as described in Fig. 1 [5]. It is well known that these second messengers are involved in modulating cellular growth and differentiation [6].

Accurate analysis of phospholipids themselves as well as measurement of enzymatic activity which is related to the phospholipids has become increasingly important, not only for studying biological processes but also for clinical implications. Recently, many approaches such as HPLC, GC and spectrophotometry for separation and quantitation of phospholipids have been developed [7–9]. One of the useful approaches involves analysis of cleavage products of phospholipids, which are generated by phospholipase C. However, these methods are experimentally complicated, relatively expensive, time-consuming and furthermore, require radiolabelled substrates.

Since the introduction of micellar electrokinetic chromatography (MEKC) by Terabe et al. in 1984 [10], this technique has been developed into very powerful and advantageous methods for the separation of various charged and uncharged compounds [11,12,20]. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF–MS), developed by Karas et al. [13], is a versatile and sensitive technique for the analysis of a variety of large biomolecules as well as phospholipids with molecular masses lower than 10^3 [21]. Since PI and DAG share comparable absorption spectra at 196 nm, spectrophotometry could not be used for monitoring breakdown of PI and formation of DAG simultaneously. However, it is possible to use the MEKC mode of CE to monitor the substrate and the product simultaneously based on a difference in partition coefficients between micellar and aqueous phases, and then use MALDI–TOF–MS to confirm the identify of the analytes.

In this study, we show that both the breakdown of substrate and formation of products of phosphatidylinositol by PI–PLC enzyme can be detected simultaneously, and the enzymatic activity can be monitored accurately and reproducibly using HPCE. MALDI–TOF–MS was used to confirm the molecular masses of substrate and product obtained after the enzyme reaction. The ability to monitor PI–PLC activity with HPCE is not only of interest from a enzymological perspective, but is also an improvement on a molecular biological technique.

2. Experimental

2.1. Chemicals and reagents

Phosphatidylinositol from bovine brain and 2,5-dihydroxybenzoic acid (DHB) for matrix using MALDI–TOF–MS were purchased from Sigma (St. Louis, USA). Chemicals used for buffers were ACS reagent grade or better. Acid and organic solvents were HPLC grade or better. PI–PLC from *Bacillus cereus* was obtained from Boehringer Mannheim (Mannheim, Germany). The water used for the matrix and sample preparations was obtained from a Milli-Q plus water purification system from Millipore (Molsheim, France).

2.2. Capillary electrophoresis and sample preparation

A P/ACE 5500 system with a photodiode array detector (Beckman Instruments, Fullerton, USA) was used for MEKC separation. Untreated fused-silica capillary tubes of 47 cm×50 μm I.D. were used. During the entire experiment, the capillary was regulated thermostatically at 40°C. Sample injections were performed by pressure (25.9 Torr; 1 Torr= 133.322 Pa) for 3.0 s. The detection wavelength was 196 nm, and the applied voltage was 17 kV. Before

the initiation of a CE run, the capillary was washed with 0.1 *M* sodium hydroxide (3 min), water (3 min) and running buffer (5 min). All the samples were passed through 0.45-μm membrane filler unit (Millex-HV 13, Sartorious, Germany) and carefully degassed before use. Data were collected and peak migration time and area were analyzed using Beckman System Gold version 8.1 software.

2.3. Incubation condition for PLC activity assay

PI–PLC was stored as a stock solution in 10 m*M* sodium azide, 10 m*M* EDTA, 50 m*M* triethanolamine at pH 7.5. The reaction mixture is prepared by mixing (in this order) 100 μl of PI suspension, 25 μl of deoxycholate solution and 100 μl of borate buffer to prevent acyl migration. The reaction is initiated by adding 2~4 μl of enzyme solution containing a 0.05~0.1 unit of PI–PLC. Assays were performed at 37°C temperature for varied time and then the enzyme reaction was terminated by boiling the sample mixture for 2 min.

2.4. MALDI TOF–MS and sample preparation

MALDI–TOF–MS analysis was performed on a HP G2025A (Hewlett–Packard, Palo Alto, USA) linear type time-of-flight mass spectrometer, equipped with a pulsed nitrogen laser (337 nm radiation). This instrument was operated in the positive-ion mode of detection. The ion accelerating potential was +28 kV and the length of flight tube was 1 m. Operating pressure for studies on the HP G2025A MALDI–TOF–MS were lower than $4 \cdot 10^{-6}$ Torr. Typically, the spectra from 60~120 laser shots were summed to obtain the final spectrum.

The MALDI–TOF matrix solution was prepared by mixing 100 m*M* 2,5-dihydroxybenzoic acid (DHB) with water–methanol (2:1, v/v). A 1-μl volume of the matrix–sample mixture was applied onto one of the ten-position gold-plated sample probes. Then the sample in the probe was crystallized with the HP G2024A sample preparation accessory. For the mass range examined in this study, the standard error in mass determination with this instrument is approximately 0.05% based on external calibration.

3. Results and discussion

3.1. Optimal conditions of CE analysis for PI and DAG

In order to monitor the PI–PLC enzymatic activity, the optimal conditions for capillary electrophoresis of PI and DAG were determined. To obtain good separation conditions for phosphatidylinositol (PI) and diacylglycerol (DAG), we investigated the effects of concentration of the running buffer, pH of the running buffer, concentration of SDS and temperature of the capillary of the PA/CE system on separation and efficiency. Upon changing those conditions, migration time and relative migration time of the analyte as well as theoretical plates per meter (*N*) were calculated as described elsewhere [14]. Standard solutions of PI and DAG were prepared by mixing the PI and DAG and diluting to appropriate concentration with 50 m*M* SDS solution. Identification of the peaks, corresponding to the individual compounds, was obtained by matching the values of relative migration time using authentic compounds. For calculating the relative migration time values, 4-acetamido phenol was used as the reference compound, which was also used as a marker for electroosmotic flow (EOF).

Fig. 2 shows an electropherogram of standard PI and DAG under the optimal conditions of 20 m*M*

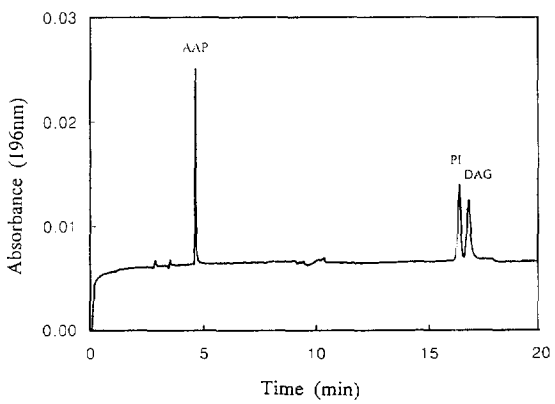

Fig. 2. MEKC separation of PLC enzyme substrate and product. Purified PI and DAG were injected by pressure for 3 s into a 47 cm×50 μm I.D. capillary and separated at 17 kV. Buffer 20 m*M* sodium tetraborate, pH 9.5, plus 150 m*M* SDS. Temperature 40°C. AAP=4-acetamidophenol, EOF marker.

sodium tetraborate (pH 9.5), 150 m*M* SDS, 17 kV at 40°C, based on the following investigations.

Borate is known to form complexes with a vicinal group including sugar or glycoprotein analogues [15]. Since the chemical structure of interest in this study contains a vicinal group on its inositol portion, we presumed that borate buffer would contribute to the separation of phosphatidylinositol and diacylglycerol, which are different only in their hydrophilic portion of the structure. Therefore, borate buffer and 20 m*M* concentration of the buffer was chosen to have a good separation and fast migration time for this study.

The presence of borate in the PI–PLC enzyme reaction mixture is also beneficial in our system to prevent acyl migration of DAG which is generated during the enzyme reaction [16]. When we used other buffer systems such as triethanolamine or 4-(2-hydroethyl)-1-piperazincethanesulfonic acid (HEPES) in the enzyme reaction, we obtained a broad peak with a minor shoulder for the peak of DAG expecting to be the mixture of 1,2-diacylglycerol and 1,3-diacylglycerol which might be the product of acyl migration. This observation is well matched with that of other investigations [16].

Since the pH of the electolyte buffer in CE analysis has a great effect on migration time of analytes through changing eletrophoretic as well as eletroosmotic flow, we examined the effect of pH of the buffer on migration time of the analytes. To study this influence in our system, we changed the pH within buffer capacity based on the pK_a values of sodium tetraborate. As pH dropped below 9.5 which is the pK_a value of borate, migration time of the analytes was increased due to reduction of zeta potential. When pH values rose above 9.5, the migration time of the analytes was also increased as shown in Fig. 3. This increase of migration time at higher pH value than pK_a, might be caused by increased eletrophoretic flow of analytes towards the opposite direction, which was the result of more negative ionization of SDS micelle and the analytes in that condition. Based on these observations, pH 9.5 of the electrolyte buffer was chosen for the rest of the study.

Separation of analytes with MEKC method using SDS can occur as a consequence of difference in partition coefficients of the analytes between micellar phase and aqueous phase. PI is less hydrophobic than DAG due to strong hydrophilic -OH groups on the inositol portion of PI, migrates faster than DAG toward the cathode and thus elutes faster than DAG. After increasing the concentration of SDS in the running buffer from 80 to 200 m*M*, separation of the two analytes of interest was better. However, above 150 m*M* concentration of SDS, the migration time and the relative migration time of both analytes increased dramatically due to increase of phase ratio, i.e., the ratio of the volume of the micellar phase to that of the aqueous phase. Therefore, we chose 150 m*M* SDS concentration in our assay system, thereafter.

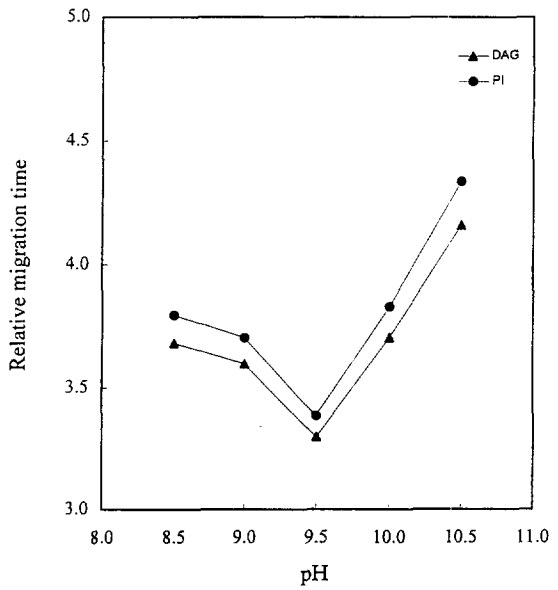

Fig. 3. Influence of pH on relative migration times of PI and DAG. Other separation conditions as in Fig. 2.

The temperature effect on theoretical plate number (N) of the analytes was also investigated. In general, the main purpose of temperature control in analysis using capillary electrophoresis is to maintain the system with relatively lower temperature to prevent excessive Joule heating which can cause band broadening. In analysis using the MEKC system, however, temperature control has another effect on separation. So, we tested the effects of four different temperatures from 20°C to 50°C under the conditions using 150 m*M* SDS on the theoretical plate number of the analytes. When we raised the temperature up

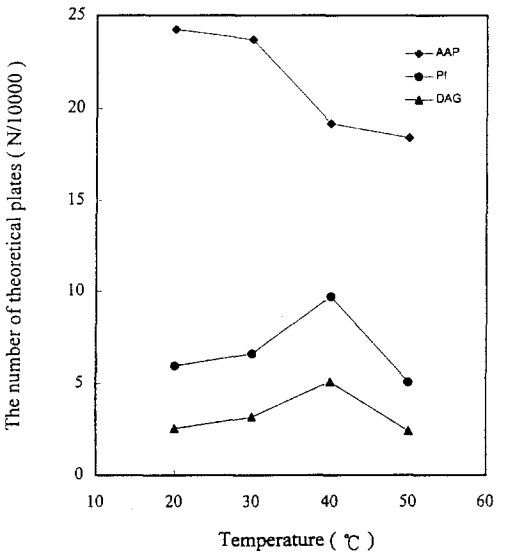

Fig. 4. Influence of temperature on number of theoretical plates per meter (N) of PI and DAG. Other separation conditions as in Fig. 2.

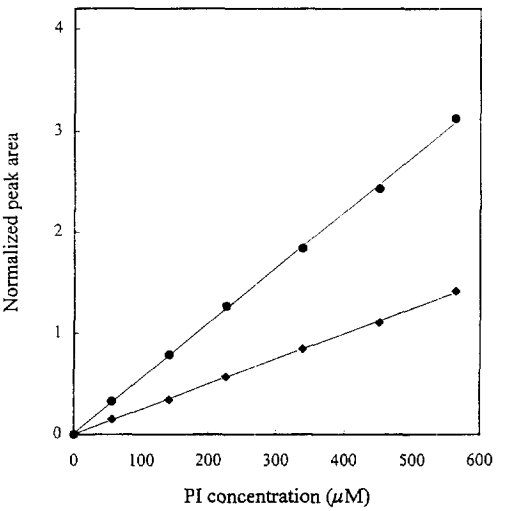

Fig. 5. Standard curves for quantification of phosphatidylinositol (●) and 4-acetamidophenol (♦). Normalized peak area of PI vs. concentration of PI was plotted. PI, $y=0.0308x+0.0120$, $R^2=0.9997$; AAP, $y=0.0140x+0.0037$, $R^2=0.9998$.

to 40°C, the theoretical plate number of both PI and DAG were increased up to $9.7\cdot10^5$ and $5.1\cdot10^5$, respectively. At a temperature of 50°C, however, the theoretical plate number of both analytes were decreased due to band broadening by Joule heat and an unbalanced thermal gradient within the capillary (Fig. 4).

3.2. Quantitative analysis of phosphatidylinositol

In order to apply the developed method of detecting PI and DAG to monitor the PI–PLC enzymatic activity practically in the future, validation of quantitative analysis of either substrate PI or product DAG is necessary. Since the response of PI to the detection wavelength of 196 nm was higher than that of DAG, we selected the substrate PI for the quantitative analysis in this study.

For quantitative analysis of phosphatidylinositol using capillary electrophoresis, we used various dilutions of PI ranging from 56.25 μM to 563.5 μM, and plotted the normalized peak area of each dilution versus the concentration of the PI. The correlation of variants, R value, was 0.9997 for the PI in the range between 56.25 μM to 563.5 μM concentrations, and the R value for the 4-acetamido phenol, which was used as an internal standard as well as the marker for electroosmotic flow, was also very good at 0.9998 (Fig. 5).

Using this calibration curve, we could determine the precise amount of PI. And, by comparing the amount of PI between the control group without the enzyme and experimental group, we could easily monitor the enzymatic activity in any system. Therefore, the observation of the good linearity in this study suggests not only the validity of the method for quantitative analysis of PI but also the feasibility of the method for monitoring the enzyme reaction.

3.3. Measurement of activity and property of phospholipase C using CE

Using the optimal conditions obtained from the above investigations, we have studied the measurement of enzymatic activity of PI-specific PLC and its dependence upon the reaction time.

Electropherograms in Fig. 6, clearly show the process of breakdown of substrate PI and formation of product DAG, dependent upon time. During the first 5 min of the reaction, 45% of the total substrate PI was consumed, and after 40 min most of the

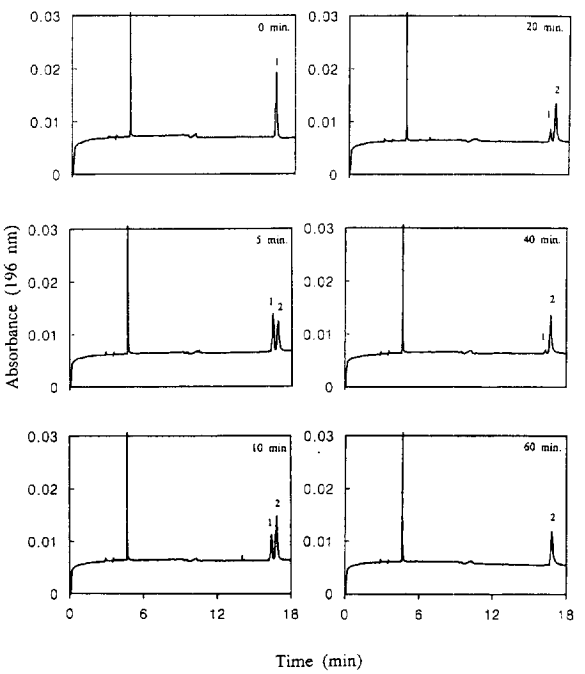

Fig. 6. HPCE analysis of the PI–PLC-catalyzed hydrolysis of PI. PI was incubated in 100 m*M* sodium tetraborate at 37°C for 0, 5, 10, 20, 40 and 60 min in the presence of 0.1 unit of PI–PLC. Peak 1, PI; Peak 2, DAG.

substrate molecule was converted to product DAG, as shown in Fig. 6.

Since we used excessive amounts of PI–PLC enzyme in the reaction mixture, loss of initial rate linearity due to depletion of substrate occurred relatively rapidly in this experiment. Therefore, for further kinetic analysis of the enzyme, if necessary, the period of linearity should be prolonged either by decreasing the amount of the enzyme to slow down the rate of product formation, or by increasing the sensitivity of the assay method, if possible. Loss of substrate was due solely to the activity of the enzyme and not to any nonspecific degradation. This was proved by having a negligible effect on the shape of the peak as well as the amount of the substrate under the conditions of prolonged incubation at 37°C for 24 h or heating at 100°C for 2 min both in the absence of the PI–PLC enzyme (data not shown).

The method developed was also used to study some properties of the enzyme, such as effects of divalent metal ions and temperature on the enzymatic reaction. Other investigators show that a chelating agent such as EDTA or *o*-phenanthroline in the PI–PLC enzyme reaction did not have any influence on the enzyme activity [17]. These findings suggest that divalent metal ions are not required for the PI–PLC enzymatic activity, which is in contrast to other forms of PLC or sphingomyelinase. Instead, the presence of divalent metal ions in the enzyme reaction showed an inhibitory effect on the enzyme activity (Fig. 7). Fig. 7 shows a plot of relative *V* (*y*-axis), which represents the normalized value of PI consumption in the enzyme reaction, versus the concentrations of the divalent metal ions $MgCl_2$, $CaCl_2$, $NiCl_2$ and $CuCl_2$. Although, all four divalent metal ions showed the inhibitory effect on the enzyme activity, the extent of inhibition was varied. IC_{50} of the divalent metal ions $MgCl_2$, $CaCl_2$, $NiCl_2$ and $CuCl_2$ on the enzyme activity were 0.56 m*M*, 0.44 m*M*, 0.20 m*M* and 0.16 m*M*, respectively.

Although, some divalent metal ions such as $MgCl_2$, $CaCl_2$, $MnCl_2$, $ZnCl_2$ were shown to have interactions with acidic phospholipids in a previous study [18], it is not clear whether the inhibitory effect of divalent metal ions is due to effects on the

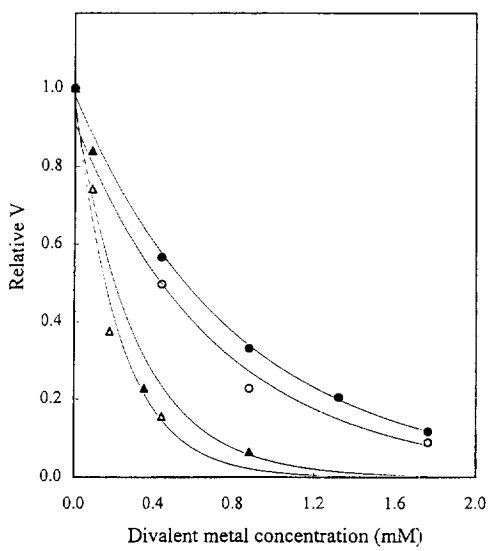

Fig. 7. Inhibition of phosphatidylinositol hydrolysis by divalent metal ions. The effects of $MgCl_2$ (●), $CaCl_2$ (○), $NiCl_2$ (△) and $CuCl_2$ (▲) were determined under standard assay conditions. PI incubated in 100 m*M* sodium tetraborate at 37°C for 30 min with a 0.05 unit of PI–PLC added into the indicated reactions to a total volume of 227 μl.

enzyme or on the substrate. There are several possibilities for the mechanism of inhibition by divalent metal ions on the activity of the PI–PLC enzyme. One possibility is that the divalent metal ions could interact with the negatively charged surface of phospholipids, which then caused the elevation of surface pH of the substrate molecules regionally [19]. Since most of the enzymes show the highest activity at neutral pH, the enzyme could be inactivated under the above condition of basic pH. A second possibility is that the divalent metal ions could disrupt an electrostatic attraction between the enzyme and the surface of the substrate molecule, which is important in enhancing the enzyme–substrate interaction.

We also studied the effect of temperature on PI–PLC activity using the CE analysis. Fig. 8 shows the electropherogram which represents the PI–PLC activity at 30, 37, 44 and 55°C. As expected and usual, the PI–PLC showed the highest activity of converting the substrate PI (peak 1) to the product DAG (peak 2) at physiological temperature. At 30°C, the enzyme activity was reduced to some extent. At high temperature, however, especially at 55°C, the enzyme activity was reduced dramatically as shown in Fig. 8. This experiment clearly shows the advantage of visualization of the CE method to study determination of activity and other properties of an enzyme.

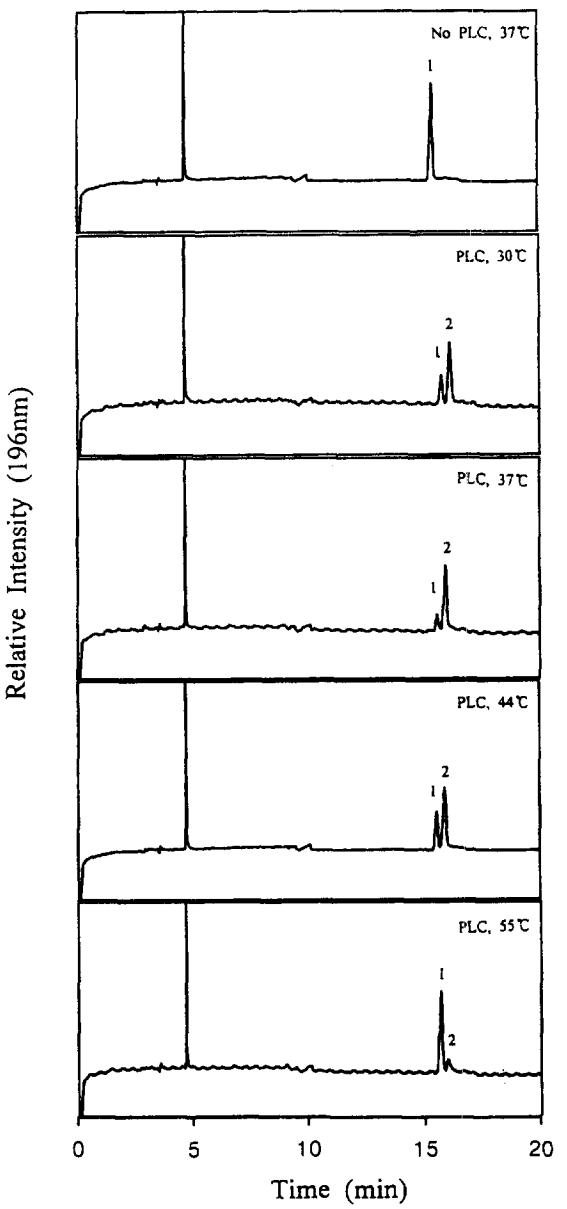

Fig. 8. Temperature-dependence of the PI–PLC-catalyzed conversion of substrate to product. PI was incubated in 100 m*M* sodium tetraborate at 30, 37, 44 and 55°C for 30 min. Other conditions as in Fig. 6. Peak 1, PI; Peak 2, DAG.

3.4. Confirmation of PI and DAG by MALDI–TOF–MS

Commercially purchased PI and DAG, originating from bovine brain was known to contain stearic and arachidonic acid predominantly. However, in many cases, the fatty acid contents of phospholipids can be varied and heterogeneous. In order to confirm the fatty acid content and exact masses of the substrate PI and the product DAG, we investigated mass analysis of the reaction mixture after enzyme digestion by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF–MS).

We initially used α-cyano-4-hydroxycinnamic acid (CHC) as the MALDI matrix. In general CHC showed the most tendency to produce strong signals and 2,5-dihydroxybenzoic acid (2,5-DHB) gave the weakest signals for phospholipids. When we used α-cyano-4-hydroxycinnamic acid as the MALDI matrix for phosphatidylinositol, we obtained strong signals but pronounced fragmentation as well. And, when we used 2,5-DHB as the matrix, we obtained

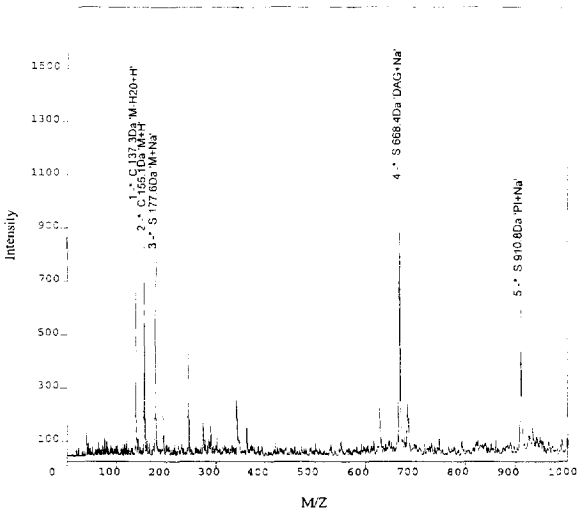

Fig. 9. MALDI–TOF mass spectra of PI and DAG. PI, M_r=887.2; DAG, M_r=645.1.

weaker but cleaner signals of molecular ions with less fragmentation. So, we decided to use 2,5-DHB as the matrix for this study.

The molecular ion peak consisted mainly of the $[M+Na]^+$ ion with a small contribution from $[M-H+2Na]^+$ and $[M+K]^+$ (Fig. 9). From this fact, we confirmed exact masses of PI (*m/z* 887.2) and DAG (*m/z* 645.1). Based on this mass analysis, we could confirm that PI and DAG contain stearic (18:0) and arachidonic acid (20:4) using MALDI–TOF–MS analysis.

4. Conclusion

In this study, we have demonstrated that both the substrate and product of the PI–PLC enzyme can be separated exactly and conveniently by micellar electokinetic chromatography analysis. Using the analysis conditions developed, we are reporting here a new approach for monitoring enzyme activity involving PI hydrolysis by PI–PLC. Loss of substrate and the formation of product in the enzyme reaction could be clearly observed simultaneously in a manner dependent upon time, various divalent metal ions and the reaction temperature. Compared to HPLC and other separation techniques, the HPCE–MEKC method is relatively rapid, reproducible and requires minimal amounts of sample and no radiolabelled substrate for detection of enzyme reaction. Moreover, the progression of the enzymatic reaction can be visualized in electropherograms with high sensitivity and reproducibility. Validity of quantitative analysis of PI using the CE method in this study also makes future study of the kinetics of this enzyme possible.

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